A Novel ε-Lysine Acylase from *Streptomyces mobaraensis* for Synthesis of *N*ε-Acyl-L-lysines

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ABSTRACT: A novel ε-lysine acylase (N_6-acyl-L-lysine amidohydrolase; EC 3.5.1.17) was isolated from *Streptomyces mobaraensis* and purified to homogeneity by SDS-PAGE from the culture broth. The purified enzyme was monomeric, with a molecular mass of approximately 60 kDa. The enzyme was inactivated by the presence of 1,10-phenanthroline and activated in the presence of Co^{2+} and Zn^{2+}. The enzyme showed a pH optimum of 8.0 and was stable at temperatures of up to 50°C for 1 h at pH 8.0. The enzyme specifically catalyzed the hydrolysis of the amide bond of various *N*ε-acyl-L-lysines. Furthermore, the enzyme efficiently catalyzed the synthesis of *N*ε-acyl-L-lysines with fatty and aromatic acyl groups in an aqueous buffer. In the syntheses of *N*ε-decanoyl-L-lysine, *N*ε-lauroyl-L-lysine, and *N*ε-myristoyl-L-lysine, the product precipitated and the yield was 90% or higher using 10 mM FA and 0.5 M L-lysine as the substrate.

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ε-Lysine acylase (N_6-acyl-L-lysine amidohydrolase; EC 3.5.1.17) has been isolated to date from animal tissues (1–3), plants (3), molds (4), and bacteria (5,6). Chibata and his coworkers (7,8) surveyed various microorganisms and isolated an ε-lysine acylase with high specific activity from *Achromobacter pestifer*. They purified the enzyme and studied its characteristics including substrate specificity (6). *N*ε-Acyl-L-lysine has recently attracted considerable attention because of its emulsifying activity, antimicrobial activity, and moisturizing function (9). In particular, *N*ε-lauroyl-L-lysine is frequently used as a component in cosmetics and lubricants (9). Currently, *N*ε-lauroyl-L-lysine is prepared by chemical synthesis (10). However, chemical methods include many tedious procedures and involve the discharge of a large amount of waste solvent. Thus, the development of an enzymatic method would be a highly desirable alternative to chemical methods. However, only one study of the enzymatic synthesis of *N*ε-acyl-L-lysine has been reported (11), in which a commercially available lipase, Lipozyme, was used in the synthesis of *N*ε-oleoyl-L-lysine from L-lysine and soybean oil in heptane at 90°C. The maximum yield was 35%.

During the course of our studies, we isolated a novel acylase from *Streptomyces mobaraensis* that specifically hydrolyzes the amide bond of *N*ε-acyl-L-lysine and could be used to synthesize *N*ε-acyl-L-lysines with fatty acyl moieties from L-lysine and a corresponding FA with quite a high yield in an aqueous buffer solution at 45°C. We purified the enzyme from the broth of *S. mobaraensis* and examined its characteristics in comparison with the enzyme produced by *A. pestifer (*6). Finally, we synthesized various *N*ε-acyl-L-lysines in an aqueous buffer solution and examined the reaction conditions for the synthesis of *N*ε-lauroyl-L-lysine.

MATERIALS AND METHODS

Materials. Beef extract, malt extract, and yeast extract were obtained from Difco Laboratories (Detroit, MI). Polypeptone was purchased from Nihon Pharmaceutical Co. (Tokyo, Japan). NZ amine (Type A) [a casein hydrolysate] and soluble starch were obtained from Wako Pure Chemical Industries (Osaka, Japan). CM Sephadex C-50, DEAE Sephadex A-50, Octyl Sepharose CL-4B, and Phenyl Sepharose CL-4B were products of Amersham Pharmacia Biotech (Uppsala, Sweden). *N*ε-Acetyl-L-lysine was obtained from Sigma-Aldrich Co. (St. Louis, MO). Various *N*α-acetyl-amino acids were obtained either from Wako Pure Chemical Industries or Sigma-Aldrich Co. L-Lysine and D-lysine were obtained from the Peptide Institute, Inc. (Osaka, Japan).

*N*ε-Chloroacetyl-L-lysine, *N*ε-benzoyl-L-lysine, *N*ε-benzyloxycarbonyl-L-lysine, *N*ε-octanoyl-L-lysine, and *N*ε-benzoyl-D-lysine, as substrates, were synthesized by reacting the copper salt of L-lysine and the corresponding acid chlorides (purchased from Wako Pure Chemical Industries) according to the method of Neuberger and Sanger (10). *N*ε-Lauroyl-L-lysine was obtained from the Ajinomoto Co., Inc. (Tokyo, Japan). Lauric acid was obtained from Sigma-Aldrich Co., and octanoic acid, decanoic acid, myristic acid, palmitic acid, stearic acid, linoleic acid, benzoic acid, and cinnamic acid were obtained from Wako Pure Chemical Industries. All other reagents were of analytical grade and were purchased either from Wako Pure Chemical Industries or Nacalai Tesque, Inc. (Kyoto, Japan).

Buffers. The following buffers were used in this study. Buffer A: 50 mM Tris-HCl, pH 8.0; buffer B: 25 mM Tris-HCl,

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pH 7.5; buffer C: 100 mM Tris-HCl, pH 7.0; buffer D: 25 mM Tris-HCl, pH 6.0; buffer E: 50 mM Tris-HCl, pH 5.0; buffer F: 100 mM Tris-HCl, pH 7.0. Buffers A–F were used, respectively, for the enzyme assay of *S. mobaraensis*, the purification of the enzyme from *S. mobaraensis*, the mobile phase of gel chromatography, the purification of the enzyme from *A. pestifer*, the enzyme assay of *A. pestifer,* and the synthetic reactions.

Cultivation of S. mobaraensis cells. Streptomyces mobaraensis IFO13819, a type culture from IFO (Institute for Fermentation, Osaka) was used in this study. The strain was aseptically transferred to an agar plate (4 g yeast extract, 10 g malt extract, 4 g glucose, and 20 g agar in 1,000 mL water, pH 7.3) and statically incubated for 7 d at 30°C. A loopful of the agar culture was then inoculated in a 300-mL shaking flask containing 30 mL of preculture medium (10 g glucose, 10 g dextrin, 5 g NZ amine (Type A), 5 g yeast extract, and 1 g $CaCO_3$ in 1,000 mL water, pH 6.5), followed by incubation at 30°C with reciprocal shaking at 120 strokes/min. A 1.6-mL aliquot of the preculture was added to a 500-mL shaking flask with baffles containing 50 mL of medium (40 g beef extract, 40 g soluble starch, 20 g polypeptone, 20 g $MgSO_4$, and 2 g K_2HPO_4 in 1,000 mL water, pH 7.0) for the main culture. The cells were grown with reciprocal shaking at 120 strokes/min at 30°C for 8 d. After cultivation, the culture broth was recovered by centrifugation (20,000 × *g*) for 30 min at 4°C.

Enzyme assays. Enzyme activity was assayed using a solution of *N*ε-acetyl-L-lysine, as the substrate, in buffer A at a final concentration of 4 mM. The reaction was carried out at 37°C, and the L-lysine produced was assayed by the acidic ninhydrin colorimetric method (12). One unit of enzyme activity was defined as the amount of the enzyme required to produce 1 μmol of L-lysine in 1 h at 37°C at pH 8.0. Protein concentrations were determined using a bicinchonic acid (BCA) Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL) with BSA as a standard.

Purification of ε-lysine acylase from S. mobaraensis. All purification procedures were carried out at 4°C. Ammonium sulfate was first added to the culture supernatant (1,470 mL) to 60% saturation to precipitate the ε-lysine acylase. The precipitate, obtained by centrifugation at 18,000 × *g* for 30 min, was dissolved in 275 mL of buffer B containing 50 mM NaCl and dialyzed 3 times against the same buffer. The dialyzed solution was placed on a DEAE Sephadex A-50 gel column (35 × 2.6 cm i.d.), which was eluted with a linear increase in NaCl concentration in buffer B from 50 to 500 mM at a flow rate of 0.35 mL/min. The active fractions were collected, concentrated, and dialyzed against buffer B containing 750 mM NaCl. The resulting enzyme solution was applied to an Octyl Sepharose CL-4B gel column (30 × 1.6 cm i.d.) and eluted with a linear decrease in NaCl concentration in buffer B from 750 to 0 mM at a flow rate of 0.3 mL/min. The active fractions were collected, concentrated, and dialyzed against buffer B containing 500 mM NaCl. The dialyzed solution was placed on a Phenyl Sepharose CL-4B column (15 × 1.6 cm i.d.) and eluted with a linearly decreasing NaCl concentration in buffer B from 500 to 0 mM at a flow rate of 0.25 mL/min. The active fractions were collected and finally dialyzed against buffer A.

Subunit M.W. measurement and SDS-PAGE. Gel chromatography was carried out on a YMC-Pack Diol-120 column (500 × 8.0 mm i.d.) to evaluate the M.W. of the protein, in which elution was done by feeding buffer C at a flow rate of 0.4 mL/min at room temperature. BSA (monomer and dimer), ovalbumin (monomer and dimer), and β-lactoglobulin were used as marker proteins.

SDS-PAGE was performed using a 12.5% gel (Mini-Protean III Ready Gels J; Bio-Rad Laboratories, Hercules, CA) by the method of Laemmli (13). Phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (LMW Electrophoresis Calibration Kit; Amersham Pharmacia Biotech) were used as standard proteins. Protein bands were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich Co.).

Effects of reagents on the stability of ε-lysine acylase. A 22.5-μL aliquot of the enzyme solution (11 μg/mL) was added to either 2.5 μL of 10 mM of reagent solutions (*p*-chloromercuribenzoic acid, iodoacetamide, DTT, glutathione, L-cysteine, 1,10-phenanthroline, and EDTA) dissolved in buffer A or 2 μL of 100 mM β-mercaptoethanol in the same buffer and pre-incubated at 37°C for 15 min. As a control, 2.5 μL of buffer A was used in place of the reagent solution. A 225-μL aliquot of the *N*ε-acetyl-L-lysine solution dissolved in buffer A was added at a final concentration of 4 mM to start the reaction, and the mixture was then incubated at 37°C for 1 h. Enzyme activity was assayed by the same method as described above.

Effects of metal ions on enzyme activity. The enzyme solution was dialyzed against buffer A containing 10 mM 1,10-phenanthroline for 4 h at 4°C to produce a metal-free enzyme preparation, followed by dialysis against buffer A for 24 h at 4°C to remove 1,10-phenanthroline. A 22.5-μL aliquot of the metal-free enzyme solution (11 μg/mL) thus prepared was then added to 2.5 μL of a 1 mM metal salt solution ($MgSO_4$, $FeSO_4$, $CaCl_2$, $AgNO_3$, $ZnSO_4$, $CuSO_4$, $CoCl_2$, $MnSO_4$, $(NH_4)_6Mo_7O_{24}$, and KCl) dissolved in buffer A and pre-incubated at 37°C for 1 h. As a control, 2.5 μL of buffer A was used in place of the reagent solution. Enzyme activity was assayed by the method described above.

pH Dependencies of activity and stability. In the following experiments, the enzyme solution, at a concentration of 10 μg/mL in buffer A containing 0.1 mM Co^{2+} (hereafter referred to purified enzyme solution) was used.

To investigate the pH dependency of enzyme activity, a 12.5-μL aliquot of purified enzyme solution was added to 112.5 μL of an *N*ε-acetyl-L-lysine solution, pH values 3.8–11.2 at a final concentration of 4 mM and incubated at 37°C for 1 h. The enzyme activity was determined by measuring the concentration of L-lysine formed, as described above.

The pH stability of the enzyme was determined as follows. A 12.5-μL aliquot of purified enzyme solution was added to 37.5 μL of 50 mM Tris-HCl buffer, pH values 3.5–12.0 and incubated at 37°C for 1 h. A 75-μL aliquot of the *N*ε-acetyl-L-lysine solution was then added at a final concentration of 4 mM

and incubated at 37°C for 1 h to determine the remaining activity.

Optimal reaction temperature and thermal stability. For the determination of the optimal reaction temperature, a 12.5-μL aliquot of purified enzyme solution was added to 112.5 μL of an *N*ε-acetyl-L-lysine solution at a final concentration of 4 mM. The reaction mixture was then incubated for 1 h at 4, 28, 37, 45, 50, 55, 60, 65, 70, and 80°C and the remaining activity determined.

The thermal stability of the enzyme was investigated as follows: A 12.5-μL aliquot of the purified enzyme solution was incubated for 1 h at 4, 25, 37, 45, 50, 55, 60, and 70°C. The treated enzyme solution was then added to 112.5 μL of an *N*ε-acetyl-L-lysine solution at a final concentration of 4 mM, followed by incubation for 1 h at 37°C, and the residual activity was then determined.

Analysis of N-terminal amino acid sequence. The enzyme was developed by SDS-PAGE as described above and then blotted electrically onto a polyvinylidene fluoride membrane (Clear Blot Membrane-P; Atto Corp., Tokyo. Japan) using a blotting apparatus (AE-6675; Atto Corp.) with blotting buffer (0.1 M Tris, 0.192 M glycine, 0.1% SDS, and 5% methanol) at 2 mA per cm^2 of a membrane for 90 min. The band stained with Coomassie Brilliant Blue R-250 on the membrane was put into a protein sequencer (Model 491; Applied Biosystems, Foster, CA).

Substrate specificity. Various *N*ε-acyl-lysines were hydrolyzed by the purified enzyme to determine its substrate specificity. A 12.5-μL aliquot of purified enzyme solution was added to 112.5 μL of substrate solution dissolved in buffer A at a final concentration of 4 mM, followed by incubation for 30 min at 37°C. The amount of L-lysine produced was determined as described above.

The hydrolytic activity with respect to various *N*α-acyl-L-amino acids was also investigated. Namely, a 12.5-μL aliquot of purified enzyme solution was added to 112.5 μL of a substrate solution dissolved in buffer A at a final concentration of 4 mM. The reaction was then allowed to proceed for 3 h at 37°C, and the concentration of amino acid released in the reaction mixture was determined by the ninhydrin method (14).

The initial reaction kinetics for the hydrolysis of *N*ε-acetyl-L-lysine was examined as follows. A 10-μL aliquot of the purified enzyme solution was added to 290 μL of a substrate solution at different final concentrations of 1–25 mM and incubated at 37°C. At appropriate times, an aliquot of the reaction mixture was withdrawn to assay the concentration of L-lysine formed. The initial reaction rate was determined from the linear portion of the plot.

Preparation of ε-lysine acylase from A. pestifer. ε-Lysine acylase from *A. pestifer* EA ATCC 23584 was purified to compare its characteristics with those of the *S. mobaraensis* enzyme. Purification was carried out at 4°C. The cells were first cultivated in the same medium as described by Ishikawa *et al.* (15). After cultivation, the cells (3.8 g in wet weight) were recovered by centrifugation and washed with a 0.9% sodium chloride solution. The cells were suspended in 35 mL of buffer D and sonically disrupted using a homogenizer (Ultrasonic Disrupter UD-201; Tomy Seiko Co. Ltd., Tokyo, Japan) at 30 W for 20 min. Cell debris was removed by centrifugation at 10,000 × *g* for 30 min followed by ammonium sulfate precipitation at 80% saturation. The precipitate was then recovered by centrifugation at 20,000 × *g* for 30 min, dissolved in 20 mL of buffer D, and dialyzed two times against the same buffer. The dialyzed solution was placed on a DEAE Sephadex A-50 column (30 × 1.6 cm i.d.) that had been equilibrated with buffer D and eluted by a linear increase in NaCl concentration in buffer D from 0 to 500 mM at a flow rate of 0.3 mL/min. Fractions showing enzyme activity were applied to a CM Sephadex C-50 gel column (30 × 1.6 cm), and elution was done under the same conditions as those for DEAE Sephadex A-50 chromatography, as described above. The active fractions were collected, concentrated, and dialyzed against 10 mM potassium phosphate buffer, pH 6.0. The solution was finally placed on a hydroxyapatite gel column (17 × 1.6 cm i.d.) and eluted by a linear increase in the potassium phosphate concentration from 10 to 300 mM at a flow rate of 0.24 mL/min. The active fractions eluted were then collected, concentrated, and dialyzed against buffer E. The specific activity of the purified enzyme was approximately 1920 units/mg, in agreement with values reported in the literature (6) in 9.0% yield, where one unit of the enzyme activity was defined as the amount of enzyme required to produce 1 μmol of L-lysine in 1 h at 42°C at pH 5.0.

Synthetic reactions of Nε-acyl-L-lysine. *N*ε-Octanoyl-L-lysine, *N*ε-decanoyl-L-lysine, *N*ε-lauroyl-L-lysine, *N*ε-myristoyl-L-lysine, *N*ε-palmitoyl-L-lysine, *N*ε-stearoyl-L-lysine, *N*ε-linoleoyl-L-lysine, *N*ε-benzoyl-L-lysine, and *N*ε-cinnamoyl-L-lysine were synthesized by a condensation reaction using 0.5 M L-lysine and the corresponding FA or aromatic carboxylic acid (10 mM) as the substrate with 10 units/mL of enzyme from *S. mobaraensis*. The condensation reaction was usually conducted in buffer F with magnetic stirring in a glass vial at 45°C. At appropriate times, a 50-μL aliquot of the reaction mixture was withdrawn for the assay of FA or aromatic carboxylic acids by HPLC with detection at 210 nm; the elution was carried out by changing the acetonitrile concentration from 35 to 90% at pH 3.0 (adjusted with 0.075% phosphoric acid as a mobile phase) at a flow rate of 0.8 mL/min. The yield was calculated on the basis of the initial molar concentration of the carboxylic acid. Furthermore, *N*ε-lauroyl-L-lysine was synthesized at different pH values and concentrations of lauric acid and L-lysine at 45°C. The enzyme from *A. pestifer* was also used in the syntheses of *N*ε-octanoyl-L-lysine and *N*ε-lauroyl-L-lysine at 45°C at pH 7.0. The final enzyme concentration was 100 units/mL. *N*ε-Octanoyl-L-lysine and *N*ε-lauroyl-L-lysine were identified by direct comparison with authentic standards using HPLC.

RESULTS AND DISCUSSION

Purification of enzyme. The enzyme was chromatographed successively into fractions on columns of DEAE Sephadex A-50 gels, Octyl Sepharose CL-4B gels, and Phenyl Sepharose CL-4B

TABLE 1
Purification Summary for an ε-Lysine Acylase from *Streptomyces mobaraensis*

Purification step	Protein (mg)	Total activity (units[a])	Yield (%)	Specific activity (units[a]/mg)	Purification (-fold)
Culture filtrate	29943	2875	100	0.096	1
Ammonium sulfate	2183	947	32.9	0.43	4.5
DEAE Sephadex A-50	299	613	21.3	2.1	22
Octyl Sepharose CL-4B	1.5	456	15.9	304	3170
Phenyl Sepharose CL-4B	0.05	169	5.9	3370	35100

[a]One unit corresponds to the amount of enzyme required to hydrolyze 1 μmol of *N*ε-acetyl-L-lysine in 50 mM Tris-HCl buffer, pH 8.0 in 1 h at 37°C.

gels. The active fractions appeared at a 280 mM NaCl concentration in buffer B from the DEAE Sephadex A-50 column. In both the Octyl Sepharose CL-4B and Phenyl Sepharose CL-4B chromatographies, the enzyme was eluted at 0 mM NaCl in buffer B. As shown in Figure 1, the active fraction collected from the final Phenyl Sepharose CL-4B column was homogeneous by SDS-PAGE, with an apparent molecular mass of around 60 kDa. Furthermore, HPLC gel filtration on a YMC-Pack Diol-120 column gave a single peak with an estimated molecular mass of around 60 kDa, indicating that the enzyme is monomeric.

The purification results for each step are summarized in Table 1. The specific activity of the purified enzyme was 3370 units/mg with a yield of 5.9%.

Some properties of the purified enzyme. Table 2 summarizes the effects of various reagents on the enzyme activity, in which the activity relative to that measured in the absence of the reagents is shown. The activity was decreased by approximately 90% by addition of 1,10-phenanthroline, indicating that the enzyme is a metalloenzyme. Furthermore, the enzyme lost approximately 85% of its activity when it was dialyzed overnight against a solution of 10 mM 1,10-phenanthroline dissolved in buffer A. However, after dialysis against buffer A without 1,10-phenanthroline, the activity was increased by 140% by addition of a final concentration of 0.1 mM Co^{2+} to the enzyme solution, as shown in Table 3. Zn^{2+} did not reactivate the enzyme at 0.1 mM, as shown in Table 3, whereas the enzyme was reactivated by 135% at 0.001 and 0.01 mM Zn^{2+} (data not shown), indicating that the enzyme is inhibited by an excess of Zn^{2+}. The concentration dependency of reactivation by Zn^{2+} was also reported for aminoacylase from hog kidney (16). The excess amount of Zn^{2+} inactivates the enzyme probably by nonspecific interaction with the enzyme. In a number of aminoacylases requiring Zn^{2+}, Co^{2+} can replace Zn^{2+} to show full activity, but Zn^{2+} seems to be bound more tightly than Co^{2+}. Thus, our enzyme probably would have a metal requirement similar to that of the aminoacylases reported previously (16,17), but further studies are necessary. In subsequent experiments, Co^{2+} was dissolved in the enzyme solution at a final concentration of 0.1 mM.

Figure 2A–D shows some selected properties of the purified enzyme. The optimal pH for the reaction is in the range of 8.0 to 9.0 at 37°C (Fig. 2A). The enzyme is stable in the pH range of 6.5 to 10.5 at 37°C (Fig. 2B) and at temperatures below 50°C for a 1-h incubation at pH 8.0 (Fig. 2C). The optimal temperature is approximately 55°C at pH 8.0 (Fig. 2D). ε-Lysine acylase from *A. pestifer* was less stable than that from *S. mobaraensis*; the *A. pestifer* enzyme lost 25 and 95% of its activity at 30 and 55°C, respectively, after 1 h.

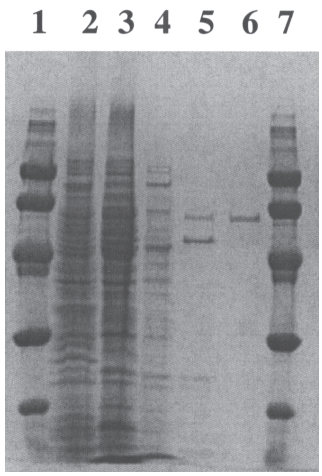

FIG. 1. SDS-PAGE of the protein fractions eluted on various column chromatographies. Lanes 1 and 7, marker proteins; lane 2, culture filtrate; lane 3, 60% ammonium sulfate precipitate; lane 4, the active fraction from DEAE Sephadex A-50 column chromatography; lane 5, that from Octyl Sepharose CL-4B column chromatography; lane 6, that from Phenyl Sepharose CL-4B column chromatography. As marker proteins, phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used.

TABLE 2
Effect of Various Reagents on Enzyme Activity

Reagents	Concentration[a] (mM)	Relative activity (%)
Control[b]	—	100
PCMB	1	86
Iodoacetamide	1	83
β-Mercaptoethanol	10	88
DTT	1	65
Glutathione	1	80
L-Cysteine	1	78
1,10-Phenanthroline	1	11
EDTA	1	75

[a]Concentration at the first incubation.
[b]The activity of the enzyme without any reagent was taken as 100%. PCMB, *p*-chloromercuribenzoic acid.

TABLE 3
Effect of Metal Ions on the Recovery of the Enzyme Activity[a]

Metal ions	Concentration[b] (mM)	Relative activity (%)
Control	—	100[c]
$CoCl_2$	0.1	141
$CaCl_2$	0.1	110
$ZnSO_4$	0.1	103
$MnSO_4$	0.1	114
$CuSO_4$	0.1	67.2
$MgSO_4$	0.1	121
$FeSO_4$	0.1	85
KCl	0.1	110

[a]The enzyme that had been first incubated in the presence of 1,10-phenanthroline was dialyzed against 50 mM Tris-HCl buffer, pH 8.0, and the metal ion was subsequently added to the dialyzed enzyme solution.
[b]Concentration in the first incubation.
[c]Activity of the enzyme dialyzed against 50 mM Tris-HCl buffer, pH 8.0 was taken as 100%. The remaining activity of apo-enzyme was 60% that of the native enzyme.

We also analyzed the *N*-terminal amino acid sequence. The first 10 amino acid residues of the enzyme from *S. mobaraensis* were SERPXTTLLR. However, we could not find any sequences with high similarity to that of the *S. mobaraensis* enzyme by the BLAST search. In addition, the sequence information on the ε-lysine acylases from other sources has not yet been reported.

Substrate specificity. Table 4 summarizes the substrate specificity with respect to various *N*ε-acyl-lysines and *N*α-acyl amino acids in terms of specific activity. The enzyme from *S. mobaraensis* appears to catalyze specifically the hydrolysis of *N*ε-acyl-lysines. It is noteworthy that the *S. mobaraensis* enzyme catalyzes the hydrolysis of various *N*ε-acyl-lysines with aliphatic and aromatic acyl moieties. Although the *S. mobaraensis* enzyme shows activity only toward *N*α-acetyl-L-lysine among *N*α-acetyl-L-amino acids, its activity is much lower than that toward *N*ε-acetyl-L-lysine; the enzyme is unable to hydrolyze *N*α-lauroyl-L-lysine. The enzyme hydrolyzes *N*ε-benzoyl-D-lysine at an extremely low rate. Interestingly, the *S. mobaraensis* enzyme is able to hydrolyze *N*ε-lauroyl-L-lysine whereas the *A. pestifer* enzyme is unable to do this, as shown below. Although the specific activity toward *N*ε-lauroyl-L-lysine shown in Table 4 is much lower than that toward *N*ε-acetyl-L-lysine, this might be due to the extremely low solubility of *N*ε-lauroyl-L-lysine. Thus, the enzyme actually would possess a much higher catalytic activity than that shown in Table 4.

ε-Lysine acylases have been isolated from a variety of sources (1–6). Although the enzyme from mammalian tissues such as rat kidney and hog kidney (1,2) shows very low specific activity toward *N*ε-acetyl-L-lysine, the bacterial enzyme from *A. pestifer* shows a level of activity similar to that from *S. mobaraensis*, as described previously. The *A. pestifer* enzyme shows a wide substrate specificity in addition to a high specific activity (6) and efficiently hydrolyzes *N*ε-acyl-L-lysines with bulky acyl moieties such as *N*ε-benzoyl-L-lysine, *N*ε-dichloroacetyl-L-lysine, *N*ε-butyryl-L-lysine, and *N*ε-octanoyl-L-lysine. The *A. pestifer* enzyme shows a considerably higher specific activity toward *N*ε-octanoyl-L-lysine than the *S. mobaraensis* enzyme, as shown in Table 4. However, the *A. pestifer* enzyme hydrolyzes *N*ε-lauroyl-L-lysine at an extremely low rate in contrast to the *S. mobaraensis* enzyme.

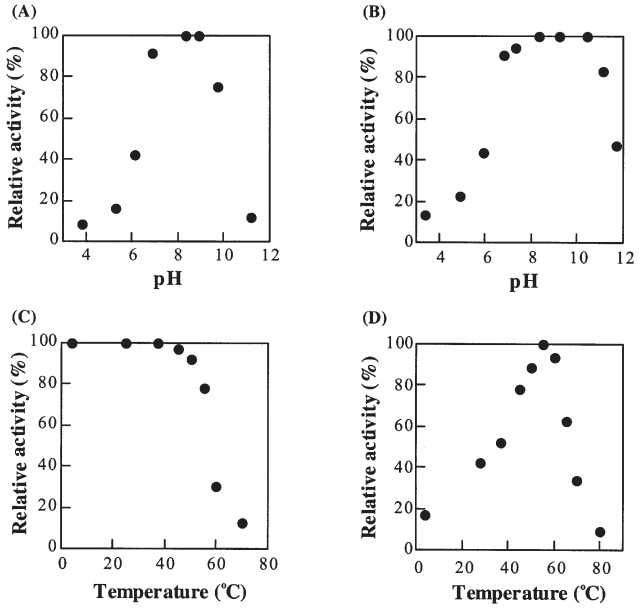

FIG. 2. Characteristics of ε-lysine acylase from *Streptomyces mobaraensis*. (A) pH Dependency of the hydrolysis activity toward *N*ε-acetyl-L-lysine at 37°C. (B) pH Stability of the enzyme incubated at 37°C for 1 h. (C) Thermal stability of the enzyme incubated at different temperatures for 1 h. (D) Optimal reaction temperature for the hydrolysis of *N*ε-acetyl-L-lysine at pH 8.0.

The substrate concentration dependency of the initial rate for the hydrolysis of *N*ε-acetyl-L-lysine using the enzyme from *S. mobaraensis* followed Michaelis–Menten kinetics (data not shown). The K_m and V_{max} values were determined to be 3.3 mM and 7860 μmol/(mg·h), respectively, whereas the K_m value for the *A. pestifer* enzyme was 0.5 mM (6).

TABLE 4
Comparison of the Substrate Specificity for ε-Lysine Acylases from *Streptomyces mobaraensis* and *Achromobacter pestifer*

Substrate[a]	*S. mobaraensis* (units[b]/mg)	*A. pestifer* (units[c]/mg)[d]
*N*α-Acetyl-L-lysine	72	0
*N*ε-Acetyl-L-lysine	3,370	2,700
*N*ε-Chloroacetyl-L-lysine	1,030	5,300
*N*ε-Benzoyl-L-lysine	1,380	15,000
*N*ε-Benzoyl-D-lysine	48	0
*N*ε-Benzyloxycarbonyl-L-lysine	220	5,200
*N*ε-Octanoyl-L-lysine	570	12,500
*N*α-Lauroyl-L-lysine	0	0[e]
*N*ε-Lauroyl-L-lysine	28	0.4[e]

[a]The substrate concentration was 4 mM except *N*α-lauroyl-L-lysine and *N*ε-lauroyl-L-lysine (2 mM).
[b]One unit was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate in 50 mM Tris-HCl buffer, pH 8.0 in 1 h at 37°C.
[c]One unit was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate in 100 mM sodium acetate buffer, pH 5.0 in 1 h at 42°C.
[d]Cited from Chibata *et al.* (6).
[e]This work.

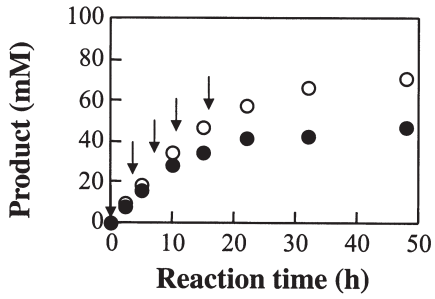

FIG. 3. Comparison of the yields of *N*ε-lauroyl-L-lysine obtained by batch and fed-batch methods. In the batch method (●), the reaction was carried out using 100 mM lauric acid and 0.5 M L-lysine. In the fed-batch method (○), the synthesis was started using 20 mM lauric acid and 0.5 M L-lysine, and lauric acid powder was added at a final concentration of 20 mM at 4-h intervals during the course of the reaction.

Synthetic reaction using ε-lysine acylase from S. mobaraensis. As described above, the ε-lysine acylase from *S. mobaraensis* isolated in this study catalyzes the hydrolysis of various *N*ε-acylated L-lysines containing aliphatic and aromatic acyl moieties. We attempted to synthesize some *N*ε-acyl-L-lysines using FA or aromatic carboxylic acids and L-lysine by an enzymatic condensation reaction. We used benzoic acid and cinnamic acid as well as FA with carbon numbers of 8 to 18 as the substrate, since the *N*ε-acyl-L-lysines formed from these FA or aromatic carboxylic acids and L-lysine would possess a variety of functions or activities, such as lubricating agents, moisturizing functions, emulsifying functions, and antimicrobial activity (9). In fact, *N*ε-lauroyl-L-lysine synthesized by chemical methods is currently on the market as a constituent of some types of cosmetics.

We conducted the synthetic reactions in buffer F at 45°C using 0.5 M L-lysine and 10 mM FA or aromatic carboxylic acids as the substrate, in which the purified enzyme was used at a concentration of 10 units/mL. Table 5 shows the yields for the synthesis of various *N*ε-acyl-L-lysines. *N*ε-Decanoyl-L-lysine, *N*ε-lauroyl-L-lysine, and *N*ε-myristoyl-L-lysine were synthesized in a yield of approximately 100%. During the syntheses of these *N*ε-acyl-L-lysines, the product precipitated from the aqueous buffer solution. Thus, the reaction was shifted to syntheses of the products with the high yields. The yields for the syntheses of *N*ε-octanoyl-L-lysine and *N*ε-linoleoyl-L-lysine are approximately 60%, which is lower than those for the syntheses of *N*ε-decanoyl-L-lysine, *N*ε-lauroyl-L-lysine, and *N*ε-myristoyl-L-lysine because of their higher solubility. The yields for *N*ε-palmitoyl-L-lysine and *N*ε-stearoyl-L-lysine are considerably low as shown in Table 5 even after a 3-d reaction, probably because the reaction rates are retarded by the extremely low solubility of the substrate FA (18). The yields for *N*ε-benzoyl-L-lysine and *N*ε-cinnamoyl-L-lysine are very low, which can be attributed to the higher solubility of the products.

We then examined the reaction conditions for the synthesis of *N*ε-lauroyl-L-lysine. The final enzyme concentration was 10 units/mL throughout the experiments. The optimal pH for the synthesis of *N*ε-lauroyl-L-lysine was approximately 7 (data not shown). At a pH value lower than 6.5, the yield was quite low. When the L-lysine concentration was changed over the range of 0.1 to 1 M, the equilibrium yield was nearly constant at 60%, but it was increased with decreasing lauric acid concentrations. When the lauric acid concentration was 10 to 25 mM with 0.5 M L-lysine, the yield was approximately 100%. However, when 50 or 100 mM lauric acid was used as the substrate with 0.5 M L-lysine, the yield after a 96-h reaction was 60%. One possible reason for the decrease in the yield with increasing lauric acid concentrations is the inactivation of the enzyme. In fact, the remaining activity after a 7-h reaction using 100 mM lauric acid and 0.5 M L-lysine was decreased to 13%. Thus, we conducted the synthesis of *N*ε-lauroyl-L-lysine starting from 20 mM lauric acid and 0.5 M L-lysine in buffer F and added lauric acid powder at a final 20 mM at 4-h intervals during the course of the reaction to prevent inactivation of the enzyme. As shown in Figure 3, both the rate of synthesis and the yield after a 48-h reaction were higher than those for the synthetic reaction starting from 100 mM lauric acid and 0.5 M L-lysine.

The enzyme from *A. pestifer* was used in the synthesis of *N*ε-octanoyl-L-lysine and *N*ε-lauroyl-L-lysine to compare it with that from *S. mobaraensis*. The reaction was carried out at 45°C in buffer F containing 100 mM lauric acid and 1 M L-lysine with a final concentration of enzyme of 100 units/mL. The yields of *N*ε-octanoyl-L-lysine and *N*ε-lauroyl-L-lysine after a 4-d reaction were 52 and 1%, respectively. The yield for *N*ε-lauroyl-L-lysine was quite low, probably because of the extremely low hydrolytic activity toward the substrate and the thermal stability of the *A. pestifer* enzyme, as described previously. The yield of *N*ε-lauroyl-L-lysine was also less than 1%, even when the synthesis was carried out at pH 5.0, the optimal pH for reaction of the *A. pestifer* enzyme.

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TABLE 5
Yields[a] of Various *N*ε-Acyl-L-lysines

Substrate	Octanoic acid	Decanoic acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Linoleic acid	Benzoic acid	Cinnamic acid
Reaction time (h)	72	24	4	24	72	72	72	24	2
Yield (%)	58.7	100	100	100	<5	<5	58.9	<1	5.2

[a]The reaction was conducted using 10 mM FA and 0.5 M L-lysine at 45°C. The enzyme concentration was 10 units/mL.

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